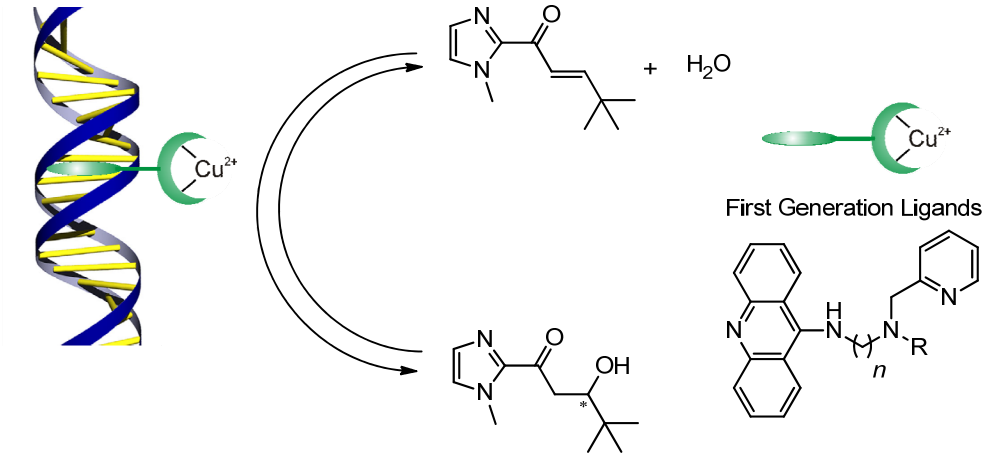
Chapter 4

DNA-based catalyzed asymmetric syn-hydration of α,β-unsaturated ketones

The enantioselective conjugate addition of water to enones in aqueous environment is a common transformation in biological systems. Recently, the first example of a non-enzymatic catalytic enantioselective hydration of enones has been presented: by using a DNA-based catalyst from ligands of the first generation ee's up to 72% were obtained for the R enantiomer of the hydration product. In this chapter efforts to provide a mechanistic understanding of the reaction are presented.



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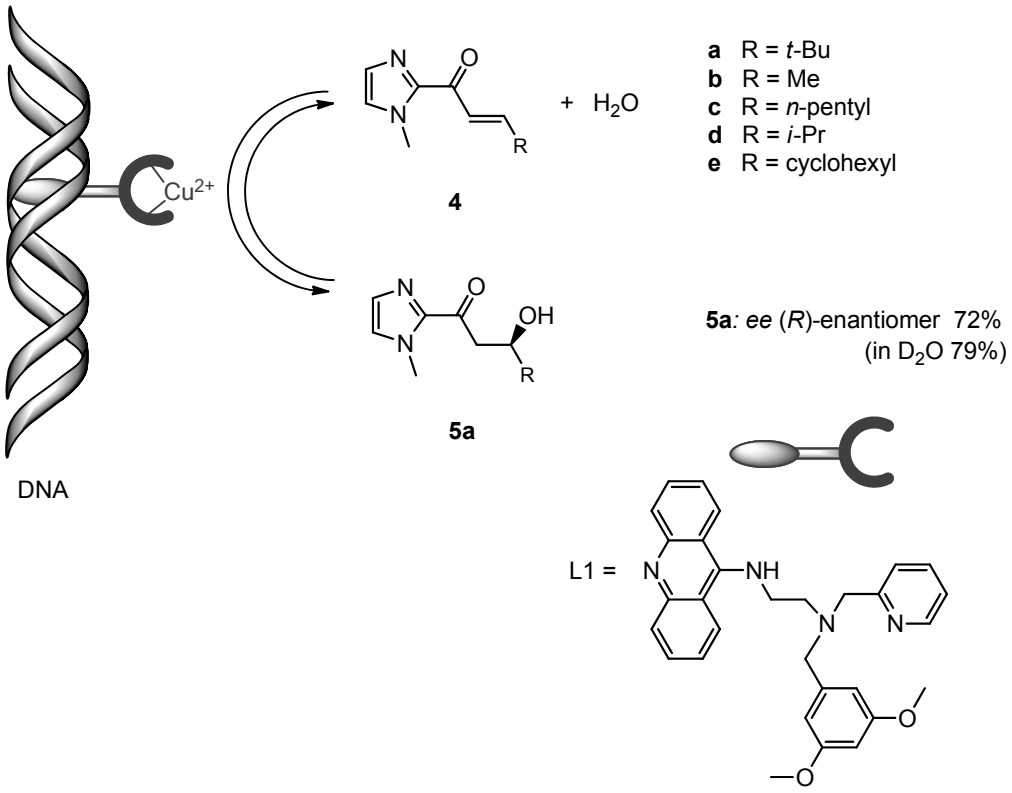
4.1 Introduction

Nature routinely uses H_2O as a nucleophile in enantioselective synthesis of chiral molecules.[1] In contrast, in synthetic chemistry, the use of water as nucleophile for conjugate addition reactions to double bonds remains a major goal, especially in aqueous media.[2-5] The asymmetric conjugate addition of water to α,β-unsaturated ketones is an important reaction which provides a chiral β-hydroxy ketone, a key structural motif in many natural products. Challenges to this transformation include the reversibility of the hydration reaction, the poor nucleophilicity of water under neutral conditions and the fact that many catalysts require anhydrous conditions in order to function. In nature, a variety of hydratase enzymes, such as fumarase and enoyl-CoA hydratase, are capable of achieving enantioselective hydration to unsaturated ketones in *anti* or *syn* selective fashion, albeit with high substrate specificity.[6-9] Recently an example of racemic enzymatic hydration of enones was presented by Hanefeld:[10] in this case, the enzyme (MhyADH) does not only catalyse the Michael addition of water to the cyclohex-2-enone substrate, but also the *in situ* oxidation of the product.

In organic chemistry, for decades the enantioselective hydration of enones has eluded the field of homogeneous catalysis. However, a number of strategies are available for the asymmetric synthesis of β-hydroxy carbonyl compounds: hydrogenation of β-ketoesters,[11] aldol reaction,[12,13] and oxa-Michael addition.[14,15] Finally, it has been reported that, by using an ill-defined heterogeneous catalyst based on wool, palladium and cobalt, α,β-unsaturated carboxilic acids could be hydrated in an asymmetric fashion.[16]

DNA-based asymmetric catalysis (Figure 1) represents an attractive solution to the difficulties of using water as nucleophile and solvent in enantioselective catalysis.[17] Our group has recently presented the first example of a non-enzymatic catalytic enantioselective *syn*-hydration of enones, using a catalyst that comprises a non-chiral Cu^{II} complex that is bound non-covalently to DNA, which is the only source of chirality present. The fact that no equivalent for this reaction exists in conventional asymmetric catalysis, shows the advantages that the DNA-based asymmetric catalysis approach has to offer.

Figure 1. Enantioselective hydration of α,β-unsaturated ketones.



4.2 State of the art

The main findings of previous studies on the DNA-based catalytic asymmetric hydration of enones were the following:[17]

- the β-hydroxy ketone can be obtained in up to 72% *ee* (79% in D_2O as medium) for the *R* enantiomer;
- the attack of the water occurs from the *Re* face of the enone moiety;
- the reaction is reversible;
- the addition of the water nucleophile to the substrate **4a** occurs in a *syn* diastereoselective fashion;
- the enantioselectivity is DNA-sequence dependent;
- an equilibrium solvent isotope effect was observed.

4.2.1 Reversibility of the hydration reaction

The *R* enantiomer of the chiral β-hydroxy ketone product (**5a**) can be obtained in up to 72% enantiomeric excess using salmon testes DNA (st-DNA) - which is natural

DNA - in combination with a Cu^{II} complex from the first generation ligand L1 (Figure 1). This was remarkable considering all the C-C bond-forming transformations previously investigated: in the Cu^{II}-catalyzed Diels-Alder,[18] Michael addition[19] reactions and in the Cu^{II}-catalyzed Friedel-Crafts alkylation,[20] the highest enantioselectivities were obtained using catalysts from the second generation ligands. Moreover, in the DNA-based catalytic asymmetric hydration the formation of the *R* enantiomer corresponds to the attack of the water from the *Re* face of the enone moiety. In contrast, in the Diels-Alder reaction[18] it was found that, by using the same catalytic system, the attack of the diene proceeded through the *Si* face of the enone moiety. The change in face selectivity suggests that in the case of the hydration reaction a different mechanism of chiral induction might be involved.

For the hydration reaction, enantioselectivity and conversion values were determined after 24 h, which has been shown the optimal reaction time for all the substrates investigated.[17] In the case of substrate **4a** (Figure 1) going to longer reaction time, the conversion value increased until 65%, where the reaction reached its equilibrium composition, but the value of enantiomeric excess (*ee*) decreased significantly until 23% for the *S* enantiomer. This was the opposite enantiomer compared to that formed in the initial stages of the reaction. The decrease in enantioselectivity and the increase in conversion was explained by considering the reversibility of the hydration reaction. In the initial stages of the reaction, the *R* enantiomer of the hydration product is formed preferentially; due to microscopic reversibility, the *R* enantiomer of the product will also be the favored substrate for the reverse reaction (dehydration reaction) from **5a** to **4a**. This will result in a decrease in the enantioselectivity once the backward reaction becomes significant. Experiments involving the pure racemic and pure *R* enantio-enriched product confirmed this hypothesis.[17] Since the *S* enantiomer is obtained in excess under equilibrium conditions, somehow a difference in free energy is induced between (*R*)-**5a** and (*S*)-**5a**: this can be tentatively explained by the formation of diastereomeric complexes of the hydration product with Cu-L1/DNA.

4.2.2 Role of Cu^{2+}/Ligand

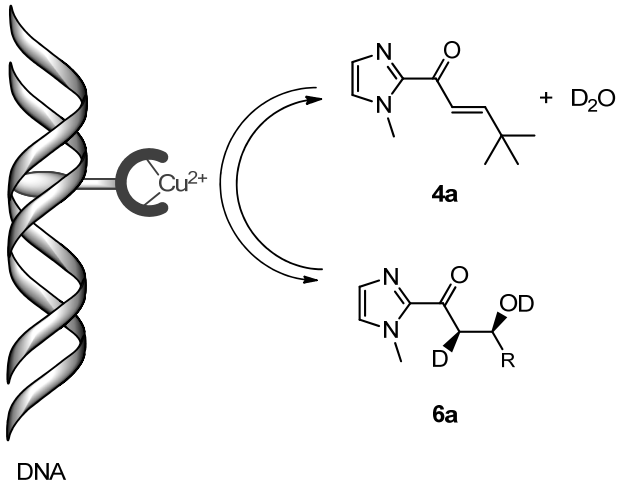
When the reaction was performed in the absence of ligand but in the presence of st-DNA and $Cu(NO_3)_2$, the *S* enantiomer of the hydration product was formed in

42% *ee*.[17] This is the opposite enantiomer compared to that obtained when the ligand is present. The results obtained with and without the ligand suggest that the Cu^{2+} ion is the catalytic species and that the combination ligand/copper ion/DNA dictates the stereochemical outcome of the reaction. The Cu^{2+} is needed for the catalysis and, since the reaction using only $Cu(NO_3)_2$ in D_2O provides the product as single diastereoisomer also, it is probably responsible for directing the hydroxyl group and the proton to the same π-face of the alkene. On the other hand, the ligand is needed to modulate the interactions with DNA resulting in the fine-tuning of the enantioselectivity, the absolute configuration and the reactivity. In fact, the catalyzed hydration reaction was found to be ligand-accelerated: after 3 h, a 36 ± 3% conversion of **4a** was observed with Cu-L1/st-DNA compared to 16 ± 1% conversion obtained in presence of $Cu(NO_3)_2$/st-DNA.

4.2.3 *Syn* diastereoselectivity

The stereochemical course of the reaction was elucidated by carrying out the transformation with D_2O as solvent (Scheme 1). It was found that the reaction in D_2O was slower than the reaction performed in H_2O but it led to higher conversion and enantioselectivity: after 3 days 40% conversion and 79% *ee* were found and after longer reaction times the conversion increased further.[17]

Scheme 1. DNA-based asymmetric *syn*-hydration reaction in D_2O.



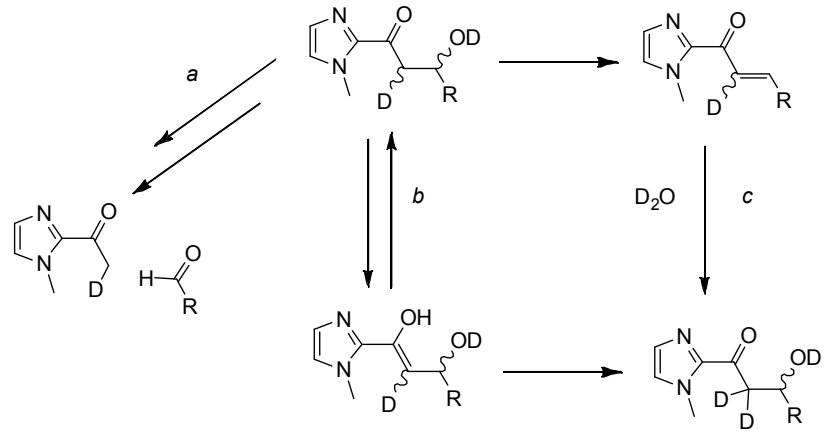
According to the ^{1}H-NMR spectrum of **6a** in $CDCl_3$[17] for the reaction conducted in D_2O, the formation of a single diastereoisomer containing one deuterium at the α carbon, occurred.[17] Based on the values of the vicinal coupling constants of the α/β-protons for the product **6a** obtained starting from **4a** in deuterium oxide as

solvent[21a] and after conformational analysis, that is well developed for aldol products,[21b] it was concluded that the product was obtained *via syn* addition of the water nucleophile to the alkene.

Interestingly, the reaction in the presence of $Cu(NO_3)_2$ alone in D_2O, that is, in the absence of DNA or ligand, also furnished **6a** as product. Hence, it was concluded that the *syn*-addition of water is not due to the presence of DNA nor to the ligand but it might be dependent on the copper catalyst or on the substrate.

The substrate with an isopropyl group on the alkenyl moiety (**4d**) gave as product **6d** as single diastereoisomer (Figure 2, a); in the ^{1}H-NMR spectrum one of the α-protons signals disappeared and the value calculated for the vicinal coupling constant between the α/β-protons was 2.0 Hz, that corresponds to the *gauche* orientation between the vicinal protons. In contrast, the presence of a small group, such a methyl, on the β-position of the acyl-imidazole (**4b**), led to the formation of the product **6b** apparently as a mixture of diastereoisomers (Figure 2, b). In this case, in the ^{1}H-NMR spectrum both signals from the α-protons are present. The *J* values between the α/β-protons were calculated to be 9 and 2.0 Hz, respectively, that correspond to the *anti* and to the *gauche* orientation of the vicinal protons. Notably, the value of the integration for the multiplet in the 4.34 - 4.24 ppm region, which refers to the β-proton, exceeds that of the combined integrals of the two α protons. This suggests that probably, for this substrate, after the time at which the reaction was analyzed (24 h), other processes start to be involved causing the full deuteration of the α-position. The full deuteration on the α-position can be a consequence of deuteration after enolization of the β-hydroxy ketone product (Scheme 2, pathway b), or of an addition-elimination process followed by a second addition of D_2O (Scheme 2, pathway c).

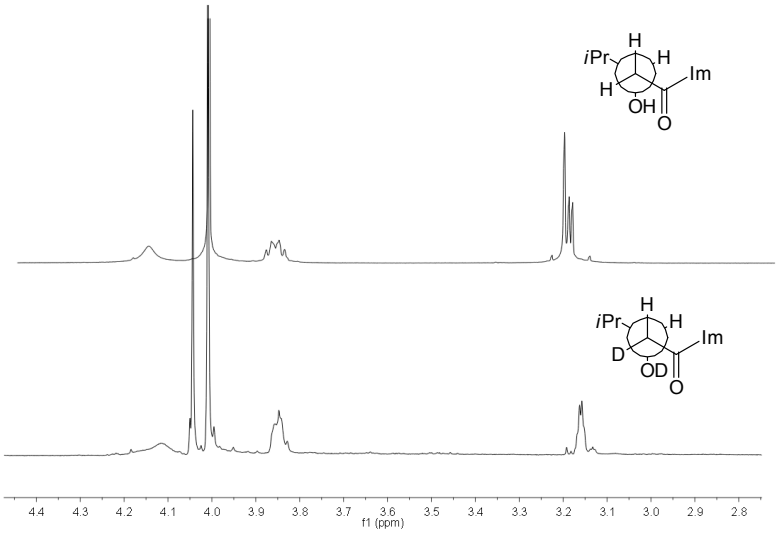
Scheme 2. Possible mechanisms involved for the hydration reaction in the presence of the substrate **4b**.



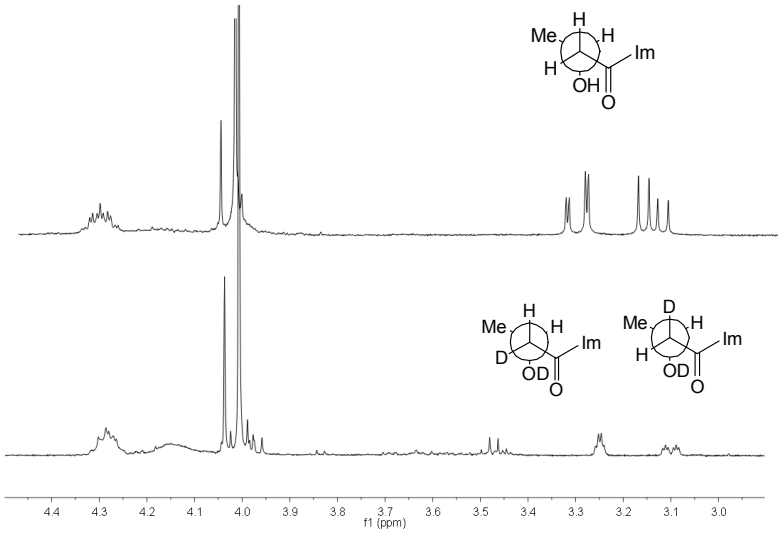
Alternatively, a retroaldol-aldol reaction with scrambling might occur (Scheme 2, pathway a) even though this possibility, based on scrambling experiment with another aldehyde (benzyl- and isopropylaldehyde), has been excluded in the case of the substrate carrying a *t*-Bu group on the β-position.[17]

Figure 2. ^{1}H-NMR of **6d** and **6b** in $CDCl_3$.

a)



b)



a) ^{1}H-NMR of **6d** in $CDCl_3$ in the region 2.75 - 4.5 ppm. The first spectrum refers to the reaction conducted in water as solvent; signals in the 3.40 - 2.98 ppm region arise from the α-protons, and signals at 3.8 - 3.9 ppm region from the β-proton. In the second spectrum, for the reaction in deuterium oxide, one of the α-protons signals disappeared; the calculated vicinal coupling constant between the α/β-protons was 2.0 Hz corresponding the *gauche* orientation of the vicinal protons; b) ^{1}H-NMR of **6b** in $CDCl_3$ in the region 2.9 - 4.5 ppm. The first spectrum corresponds to the hydration product obtained in the reaction conducted in presence of Cu/L1 complex in water as medium; the signals in the 3.35 - 3.05 ppm region correspond to the α-protons; the multiplet in the 4.35 - 4.35 ppm region corresponds to the β-proton. The second spectrum refers to the reaction conducted in deuterium oxide as medium.

4.3 Isotope Effect

The observation of isotope effects can be helpful for the postulation of a plausible mechanism of a chemical reaction. The introduction of deuterium in place of hydrogen and its exchange with water in some sites of enzymes and substrates might cause solvent isotope effect on the kinetic and equilibrium constants of the reaction investigated.[22a-g] A kinetic isotope effect is defined as the ratio of the rate constants for the reaction with the natural and the altered isotope, respectively; the change in rate arises from the increase in the activation energy due to the replacement of an atom by a heavier isotope. From the magnitude of the isotope effect it is possible to understand whether the bond, at which the isotopic substitution occurs, changes during the rate determining step: if the ratio k_H/k_D is greater than 1 the isotope effect is defined '*normal*'; in the opposite situation, it is defined '*inverse*' while if the ratio does not differ from unity it can be concluded that the bond where the substitution with the heavier atom occurred is not changing during the rate limiting step (or the effect too small to be detectable). An additional distinction is between primary and secondary isotope effect: in the first case the bond breaking event occurs at the X-H/X-D bond; in the other, the bond involved in the isotopic substitution is not broken nor made in the rate determining step of the reaction but it is attached to an atom undergoing a change in hybridization.[22h]

The synthetic challenges to introduce an isotope into a specific position in a molecule can be overcome by stirring the reactant in a deuterated protic solvent when the position to be deuterated is readily exchangeable with the solvent; in this way, the reactant will be deuterated.[22h] This allows for observation of solvent isotope effect which can influence equilibria or rates compared to non-deuterated solvents. In the case of the hydration reaction, by using deuterium oxide as solvent the reaction is slower compared to the reaction conducted in pure water (after 15 days the reaction with the substrate **4a** reached 54% conversion and 72% *ee*). Hence, the lower conversion at similar reaction times can be attributed to a kinetic/solvent isotope effect.

The hydration reaction is considered pseudo first-order, as the water concentration is constant.[17] The experiments to determine the isotope effect imply performing the reaction in both water and deuterium oxide as solvent independently; a small excess of Cu^{2+} was used so that the K_d referring to the dissociation of the product from the catalyst could be reduced and considered

negligible. The samples were analyzed by HPLC; this choice was made because of complications occurring during the spectrometrically detection which are caused by the absorption of the acridine complex in the same region as the substrate and the product of the reaction. The values of the observed apparent rate constants measured for the hydration reaction performed in H_2O and D_2O were $7.33 \times 10^{-5} \pm 3.74 \times 10^{-6}$ s^{-1} and $1.82 \times 10^{-5} \pm 2.47 \times 10^{-7}$ s^{-1}, respectively. The resulting isotope effect was calculated to be 4 (normal isotope effect). That proves that the substitution H/D occurs at the bond that is involved (broken or created) during the rate determining step of the reaction.

4.3.1 Proton inventory

The proton inventory technique is based on the fact that the solvent isotope effect k_H/k_n, for a variety of reactions in H_2O-D_2O mixture is a function of n, the molar fraction of deuterium in the mixture.[22h] The dependence of the KSIEs on n is represented by the Gross-Butler equation:[22h]

$$k_n/k_o = \frac{\Pi^{TS}(1 - n + \Phi^{T}_{i}n)}{\Pi^{TS}(1 - n + \Phi^{R}_{j}n)} \qquad (1)$$

$$k_n / k_o = \Pi^{TS}(1 - n + \Phi^{T}_{i}n) \qquad (2)$$

$$k_n / k_o = (1 - n + \Phi^{T}_{i}n)^2 \qquad (3)$$

where k_n and k_o are the rate constants for a solvent whose D atom fraction is equal to n, and for pure water; Φ refers to the fractionation factor for every proton which exchanges with the solvent during the process under study; the superscripts R and T refer to reagents and transition state, respectively; n is the molar fraction of deuterium in the solvent and varies from 0 (pure H_2O) to 1 (pure D_2O). A common assumption is that there is no contribution of the reagent to the observed isotope effect so that the KSIE is entirely generated by the Φ^T(eq. 2).[22b] The shape of the resulting plot of the activity, expressed as the ratios between the rate constants in the mixtures (k_n) and in pure water (k_o), versus the mole fraction of D_2O (n), gives information about the number of protons which are transferred during the rate determining step of the reaction. The most simple case occurs when a straight line is obtained in the graph: this means that the activity decreases proportionally with the increase of the mole fraction of D_2O and that only one

proton is transferred in the rate determining step of the reaction. Curved lines are obtained when more than one proton is involved and the exact number can be determined sometimes on the basis of the shape of the graph, even though the interpretation can be uncertain. After determining the kinetic parameters of interest in a number of isotopic water mixtures of deuterium atom fraction *n*, the data can be fit to obtain the value of fractionation factors (Φ^T and Φ^R; fractionation factor defined as inverse of the isotope effect $\Phi = k_n/k_H$). In our case, a proton inventory experiment was performed and the results are summarized in Table 1 and depicted in the graph shown in Figure 2.

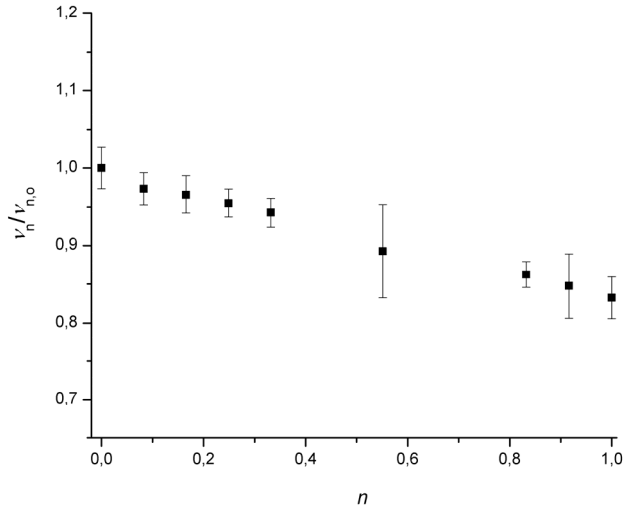
Table 1. Dependence of the ratio $v_n/v_{n,0}$ from *n*.

n	Rates ($M \cdot s^{-1}$) $\times 10^{-9}$	$v_n/v_{n,o}$
0	1.84 ± 0.034	1
0.082	1.79 ± 0.017	0.973
0.165	1.78 ± 0.029	0.965
0.248	1.76 ± 0.079	0.955
0.331	1.73 ± 0.013	0.942
0.551	1.64 ± 0.106	0.892
0.832	1.59 ± 0.007	0.862
0.916	1.56 ± 0.070	0.847
1	1.53 ± 0.040	0.832

The results are an average of two experiments

According to these data, the shape of the graph is linear which implies that only one proton is involved in the rate determining step of the hydration reaction. A Φ value of 0.84 was calculated. Much poorer fits were obtained for two- and infinite-sites models.

Figure 2. Plot of $\nu_n/\nu_{n,0}$ versus *n*.



4.3.2 Discussion

Useful information about the mechanism of the DNA-based Cu^{II}-catalyzed asymmetric conjugate addition of water to enones can be gained from a comparison to the data available in literature for the hydratase-dehydratase enzymes which operate *via* an *anti* or *syn* pathway.

In general, non-enzymatic addition/abstraction reactions favor *anti*-elimination because of a combination of stereoelectronic[23] and steric effects since the *syn*-reactions would involve a high energy eclipsed geometry in the transition state. Similarly, a group of enzymes (fumarase hydratase, enolase and aconytase hydratase) proceed in an *anti* fashion.[24,25a] In the case of *anti*-elimination, usually, the proton abstracted is in α position to a carboxylate group. At the opposite, when the proton abstracted is in α to a carbonyl group of a thioesther or a ketone[25] *syn*-pathways are favored;[26,27] some examples of the latter case, are represented by Enoyl-CoA hydratase,[26] fatty acids synthetase and β-hydroxydecanoyl thioesther dehydratase.[24,25a]

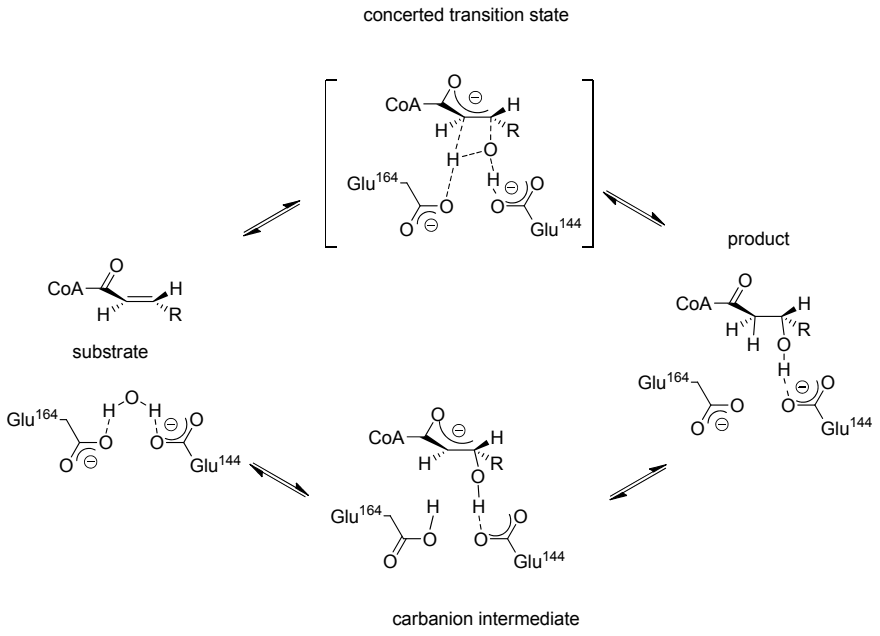
Because of microscopic reversibility, hydration and dehydration reactions will proceed *via* the same mechanism. Dehydration (elimination) reactions catalyzed by enzymes may proceed in a stepwise pathway (E1cb) which involves a carbanion species or, alternatively, without carbanion but with electrophilic catalysis.[28] Another possible mechanism is the concerted elimination (E2) with or without electrophilic catalysis. The transition from the concerted E2 mechanism to the

E1cb pathway depends on the stability of the carbanion: if the stability of the carbanion increases, the E1cb mechanism is favored.[29]

The enzymatic *anti*-elimination reactions examined so far have been shown to proceed in a stepwise pathway[23] through the intermediacy of an unstable carbanion species (E1cb). Instead, for the Enoyl-CoA hydratase, which acts via a *syn* pathway, it has been shown that the reaction may proceed either in a concerted[27,28] or a stepwise pathway[30] in which all the added atoms come from the same water molecule; the molecule of water in this case was suggested to be held in place by two glutamate residues in the active site acting as base/acid (Figure 3).

Kinetic isotope effect experiments should help to address the question whether a stepwise or concerted mechanism is involved. In our case, the proton inventory experiment suggests that only one proton is involved in the rate determining step of the hydration reaction. Previously in this chapter, it has been shown that substrates carrying a bulky substituent on the β-position (like *t*-Bu (**4a**) or *i*-Pr (**4d**) groups) undergo the conjugate addition of water in a *syn* diastereoselective fashion.

Figure 3. Proposed mechanism for Enoyl-CoA.

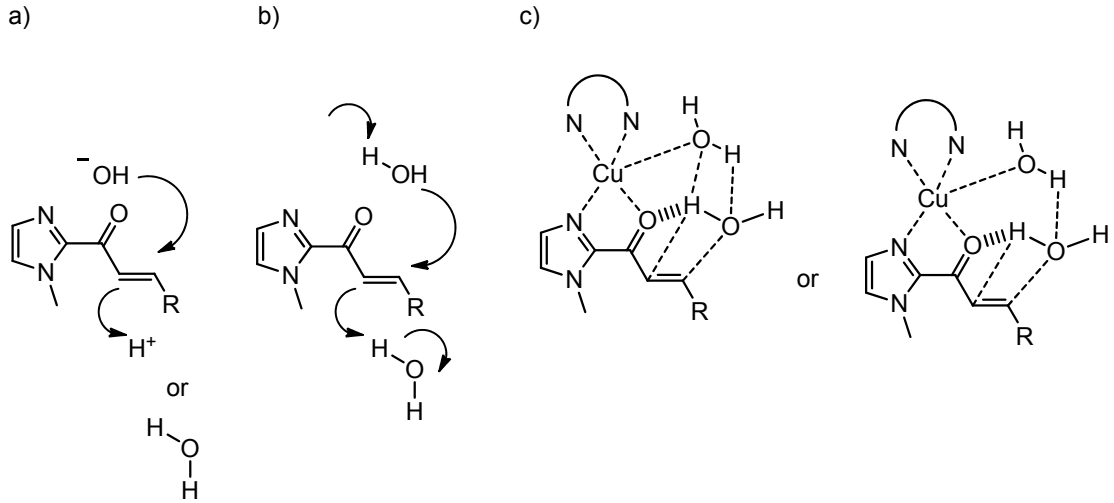


For these substrates the stepwise mechanism is not likely: indeed, the formation of a planar carbanion which is involved in the Ec1b mechanism, would proceed in a non-selective fashion since the attack of the proton could occur from both sides of the enolate. In this case, it is likely that a concerted mechanism occurs (Figure 4).

However the first mechanism depicted in Figure 4a can be discarded because of the low concentration of $^{-}$OH ions at the pH which the reaction is performed at (pH = 5.5); the second scenario, depicted in Figure 4b, is also unlikely since it would imply two protons moving in the rate determining step, which is in disagreement with what was found in the proton inventory experiment. The most probable mechanism would be that depicted in Figure 4c. In our case, considering that also copper alone gives *syn* addition, DNA and ligand can be considered not responsible for the diastereoselectivity observed. Hence, instead of two assisting groups holding in place the water molecule, as suggested for the enoyl-hydratase, it is likely that water coordinated to the copper ion would assist the addition of water to the double bond.

An exception could be represented by the substrate in which a methyl group is present on the β-position (**4b**). In this case, the reaction led to the formation of both diastereoisomers. This would suggest or a different mechanism occurring (stepwise) or a concerted mechanism in which the presence of a small substituent still allows for rotation of the bond or for the attack of the counterion from both sides of the enone. However, the observed loss of diastereoselectivity may be also due to the fact that the reaction was analyzed after the equilibrium was reached.

Figure 4. Hypothetical mechanism of the hydration reaction.



However, the results of the proton inventory are never completely unambiguous[22b] and based on these data it can be only suggested that one exchangeable site is involved in the rate determining step of the reaction. Additional experiments should be performed in order to obtain further information on the mechanism of the reaction.

4.4 Effect of the DNA-sequence

Spectroscopic data and high resolution crystal-structure have shown sequence-specific variations in the structure of the DNA double helix and sequence-dependence interactions with cations and water.[31] These local variations in the structure of the DNA and/or in solvation of the double helix are crucial for DNA recognition by enzymes, transcription factors and small molecule ligands.[32] In our case, it is likely that the outcome of the reaction catalyzed by the Cu^{II} complex depends on those variations as well. In fact, in DNA-based asymmetric catalysis, it has been found that the DNA-sequence significantly affected the reactivity and the enantioselectivity of the reaction under study.[20,32,33]

For the hydration reaction, initial studies showed that oligonucleotides containing an AT central segment gave the best result in terms of reactivity and enantioselectivity.[17] In this study, a broad range of sequences differing from each other in the content of AT bases, were analyzed. Initially this was done for the reaction performed in H_2O as medium; once the segments in the sequence that seemed to be responsible of higher *ee's* values were identified, a further optimization was conducted which involved performing the reaction with selected oligonucleotides in D_2O. This should give raise to higher enantioselectivities (*vide supra*; Table 2).

Conversion and enantioselectivity values were determined after 24 h. The reactions were performed with the Cu-L1/DNA complex and the substrate (**4a**) (which gave the highest enantioselectivity in the presence of st-DNA).[17] As previously observed,[17] it was found that, by performing the reaction in D_2O, the conversions were lower than in H_2O and the enantioselectivity values were higher. Sequences rich in GC bases (Table 2, entries 1-6) gave lower enantioselectivities in both H_2O and D_2O compared to those obtained by using sequences with high AT content (entries 7-16). This is remarkable since with the same Cu-L1/DNA complex in the Diels-Alder reaction, a clear preference for sequences containing GC segments was observed.[34] This might be due to a different preferential positioning of the complex within the DNA depending on the reaction involved (*vide infra*). The best result in terms of enantioselectivity for the hydration reaction was obtained with the dodecamer CAAAAATTTTTG, which gave up to 82% *ee* for the *R* enantiomer of the hydration product (entry 10).

Table 2. DNA-sequence dependence of the hydration of **4a** catalyzed by DNA/Cu-L1 in H_2O and D_2O.[a]

entry	DNA-sequence	water		deuterium oxide	
		conversion[b]	*ee*[b]	conversion[b]	*ee*[b]
1	d(CGCGGGCCCGCG)$_2$	15	41		
2	d(CGCGCCGGCGCC)$_2$	20	15		
3	d(CGCGCGCGCGCG)$_2$	20	50	10	59
4	d(CGCGCGCGCGCG)$_2$			38[c]	42
5	d(GCGCGCGCGCGC)$_2$			8	50
6	d(GCGCGCGCGCGC)$_2$			33[c]	40
7	d(ATATATATATAT)$_2$	58	60	20	76
8	d(TATATATATATA)$_2$			38	67
9	d(AAAAAATTTTTT)$_2$	38	52		
10	d(CAAAAATTTTTG)$_2$			16	82
11	d(TAAAAATTTTTA)$_2$			22	77
12	d(CCCAAATTTGGG)$_2$	47	55	19	75
13	d(GCAAAATTTTGC)$_2$	37	61	20	75
14	d(CGCAAATTTGCG)$_2$	38	62	20	77
15	d(CGCAAATTTGCG)$_2$			14[c]	62
16	d(CGCGAATTCGCG)$_2$	37	63	15	72
17	d(CGCGATATCGCG)$_2$	46	63	20	78
18	d(CGCGATATCGCG)$_2$			14[c]	70
19	d(CGCGTATACGCG)$_2$	40	55	24	78
20	d(CGCGTATACGCG)$_2$			29[c]	66
21	d(GCGCTATAGCGC)$_2$			22	79
22	d(TCGCTATAGCGA)$_2$			16	80
23	d(CGTCTATAGACG)$_2$			16	79
24	d(TCAGTATACTGA)$_2$			19	80
25	d(CGCATATATGCG)$_2$	45	58	15	72
26	d(CAACAATTGTTG)$_2$			23	80
28	d(CTAGCATGCATG)$_2$			11	66
29	d(CACACATGTGTG)$_2$			22	79
30	d(CAACTATAGTTG)$_2$			24	81
31	d(CACATATATGTG)$_2$			25	79
32	d(TCGAGTATACTCGA)$_2$			26	79
33	d(AGTAGTATACTACT)$_2$			22	77
34	d(AGTACTATAGTACT)$_2$			23	78
35	d(TCGACTATAGTCGA)$_2$			18	76
36	d(AGTATACT)$_2$			18	66
37	d(ACTATAGT)$_2$			21	75
38	d(CACATGTG)$_2$			26	74
39	d(AAAATTTT)$_2$			21	65

[a] Conditions: see experimental section. [b] *ee* and conversion values were detected by HPLC (see experimental section); *ee* values refers to the *R* enantiomer of the product in all the cases and they are an average of at least two experiments; they are reproducible within 2%. [c] 100 mM NaCl.

In general, the presence of the central segment ATAT/TATA resulted in a high enantioselectivity value (entries 17-25; 30-32). The presence of NaCl did not have a beneficial effect on the enantioselectivity (entries 4, 6, 15, 18, 20); a significant increase in conversion (20%) was observed with GC/CG sequences (entries 4, 6).

By varying the number of consecutive A/T bases within the helix (entries 9-16), the *ee* values did undergo a significant but not dramatic decrease (from 82% to 72%, entries 9-16). Moreover, upon modification of the bases in proximity of the TATA central segment, no significant changes in enantioselectivity occurred (entries 19, 21-25). Finally, small variations in the length of the oligonucleotide (14-mer, 8-mer) were considered: with 14-mers containing the TATA central segment the values of enantioselectivity were in the same range (entries 32-35) compared to those found in presence of the dodecamers, albeit in the same time, slightly higher conversion was observed; upon shortening of the sequences to eight base pairs, a drop in enantioselectivity was detected (entries 36-39).

Even though sequences which allowed for slightly higher enantioselectivities with respect to the reaction performed in the presence of salmon testes DNA (st-DNA) were found, it is still not possible to identify unequivocally the crucial requirements of the DNA composition to achieve high reactivity and selectivity. A tentative explanation is provided later in this chapter.

To understand to which extent the DNA-microenvironment affects the enantioselectivity in the hydration reaction once the equilibrium is reached, the values of enantiomeric excess were detected after reaction times longer than 24 h in D_2O for the substrate **4a**. The results for the reaction in the presence of synthetic oligonucleotides after 20-30 days, are reported in Table 3.

By performing the reaction in H_2O, it was observed that in the presence of Cu/L1-DNA after 15 days, 68% of conversion to the product **5a** was reached and the *S* enantiomer was formed in 23% *ee*. Instead, the reaction in the presence of st-DNA/Cu-L1 in D_2O after the same time gave 55% conversion and 72% *ee* showing that the reaction is slower in D_2O; after 30 days the conversion increases further to 66% and the *ee*, which still refers to the *R* enantiomer of the product, decreases. For all the entries in the presence of synthetic oligonucleotides (12-mer) the *ee'*s values were significantly lower after reaction times longer than 1 day: a decrease of 20-30% compared to the initial stage of the reaction was observed. In general, the values of conversion observed did not exceed 72%. Notably, in all these cases

still the *R* enantiomer of the product was observed in excess which suggests that the racemization process is much slower in D_2O.

Table 3. *Ee* at equilibrium for the hydration reaction of **4a** in D_2O with synthetic oligonucleotides in the presence of Cu/L1.[a]

entry	sequence	time (days)	conversion (%)[b]	*ee* (%)[b]
1	St-DNA	1	12	84
2		15	55	72
3		30	66	58
4[c]		1	<5	73[d]
5[c]		15	22	43[d]
6[c]		30	45	38[d]
7	$d(GAAAAATTTTTC)_2$	1	16	63
8		30	68	49
9	$d(TAAAAATTTTTA)_2$	1	22	77
10		30	67	47
11	$d(GCGCGCGCGCGC)_2$	1	8	50
12		30	58	48
13	$d(GCGCTATAGCGC)_2$	1	24	78
14		30	67	54
15	$d(TATATATATATA)_2$	1	38	67
16		30	74	40
17	$d(CCCAAATTTGGG)_2$	1	19	75
18		30	71	51
19	$d(CGCGAATTCGCG)_2$	1	15	72
20		30	71	46
17	$d(CAACAATTGTTG)_2$	1	23	80
18		22	72	50
19	$d(CTAGCATGCATG)_2$	1	11	66
20		22	62	56
21	$d(CACACATGTGTG)_2$	1	22	79
22		22	67	50

[a] Conditions: see experimental section. [b] *ee* and conversion values were detected by HPLC (see experimental section); *ee* values refers to the *R* enantiomer unless noted otherwise; values are an average of at least two experiments and are reproducible within 2%. [c] $Cu(NO_3)_2$ (no ligand). [d] *S* enantiomer formed.

4.4.1 DNA-sequence selectivity for the hydration reaction in the absence of the ligand

Nucleic acids, because of their negative charges, are excellent targets for metal ions since they provide a large choice of binding sites including phosphate oxygen atoms, the atoms from heterocyclic nucleobases and in part, the hydroxyl groups

of the sugars.[33] In the absence of ligand, it is likely that Cu^{2+} would have different locations within the DNA helix. Hence, we investigated the dependence of the enantioselectivity on the DNA-sequence for the reaction performed in the presence of $Cu(NO_3)_2$ alone and a small series of synthetic self-complementary oligonucleotides. It was found that $Cu(NO_3)_2$ bound to defined sequences (see Table 4) gave values of enantioselectivity up to 42% even though the differences among the sequences screened are not dramatic; moreover in all cases examined, the enantioselectivity values were lower with respect to the situation in which $Cu(NO_3)_2$ is used in combination with st-DNA.

Table 4. Dependence of the enantioselectivity on the DNA-sequence for the hydration reaction conducted in the absence of ligand. [a]

entry	DNA sequence	conversion (%)[b]	*ee* (%)[b]
1[c]	st-DNA	20	42 (*S*)
2	$d(TATATATATATA)_2$	17	32 (*R*)
3	$d(CAAAAATTTTTG)_2$	11	16 (*R*)
4	$d(TAAAAATTTTTA)_2$	12	8 (*R*)
5	$d(GCGCGCGCGCGC)_2$	8	9 (*S*)
6	$d(CACACATGTGTG)_2$	6	13 (*S*)
7	$d(CGCGGGCCCGCG)_2$	3	18 (*S*)
8	$d(GCGCTATAGCGC)_2$	7	11 (*S*)

[a] Conditions: see experimental section; reaction times 2 days, D_2O. [b] *ee* and conversion values were determined by HPLC (see experimental section); *ee* values are an average of at least two experiments; they are reproducible within 3%. [c] 1 day.

The values of conversion are generally low after 2 days and this is in agreement with the previous finding that the reaction is ligand accelerated (*vide supra*). The best result so far was obtained by using the dodecamer AT (Table 4, entry 2). There is a different correlation between *ee* and DNA composition compared to what observed for the reaction conducted in the presence of ligand: sequences that gave the highest enantioselectivities before, in this case gave only modest *ee* (*e.g.* entries 3, 4, 6, 8).

As previously mentioned, the reaction performed in the absence of ligand but in the presence of st-DNA and $Cu(NO_3)_2$ in water leads to the formation of the *S* enantiomer of the hydration product in 42% *ee*.[17] Notably, with some sequences

the formation of the *R* enantiomer was observed. A detailed spectroscopic study is required to understand the reasons of the change in enantio-preference.

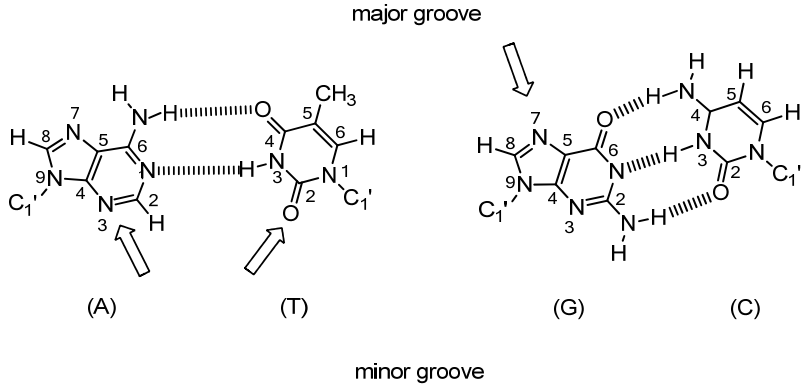
4.4.2 Discussion

Although the Watson-Crick base pairs do not have any formal overall charge, they contain both acceptor and electron donor regions; hence, the functional groups present in the minor and major grooves determine an electrostatic surface potential which is sequence-dependent. This leads to non-uniform localization of bivalent cations.[35] To explain the preference of the Cu/L1-complex and of the Cu^{2+} alone for the A·T bases in the hydration reaction, it is hypothesized that the spine of hydration plays an important role in directing the complex in the narrower minor groove of the A·T base pairs where two sites are available for coordination. For direct cation interactions (after partial dehydration) or indirect interaction (via shared water molecules of hydration that bridge DNA and cations), the A·T base pairs have two electronegative groups (Figure 5) in their minor groove side, that is the N3 of adenine and the O2 of thymine; hence, the A·T base pair is expected to localize preferentially cations in the minor groove. In the G·C pair the amino group (which has a positive electrostatic potential) between the N3 of guanine and the O2 of cytosine would interfere with any cations localization; in the major groove, the most favorable sites for cation interactions are the N7, O6 electron donor atoms of guanine compared to the N7 of the A which has adjacent N6 amino group. Thus DNA-cation interactions may occur in the minor groove of the A·T base pairs and in the major groove in GC-rich regions.

The different enantioselectivity observed, by using the same catalyst and defined oligonucleotides, in the Diels-Alder and in the hydration reactions can be tentatively explained with the fact that the G·C base pair is less involved in the hydrogen-bond network formed in the first and second hydration layers[35e] which is useful for indirect cations interaction. Hence, in the hydration reaction, assuming the water bridges between DNA and cations are responsible for directing the attack of the water molecule on the enone substrate once the complex is accommodated in the helix, the complexes residing in minor groove of the A·T rich regions might contribute, to a bigger extent, to the overall enantioselectivity. Albeit the acridine-based complex - depending on the π-π interactions of the acridine moiety with DNA bases and on the specific interactions the spacer and metal binding moiety of

the specific ligand molecule might have with DNA - would preferentially reside in the GC rich regions.[36]

Figure 5. Chemical structures of the A·T and the G·C base pairs and available sites for metal coordination.



The preferential sites for coordination to the metal in both minor and minor groove are indicated by the arrows.

However, for the hydration reaction there is not a clear trend and there are no dramatic differences in the values of enantioselectivities among the sequences tested; this can be due to the fact that several binding modes of Cu^{II} complex to the DNA exist and the observed enantioselectivity is an average of the contribution of all the copper complexes located within the helix.

Apparently, also in the presence of the Cu^{2+} alone, higher enantioselectivities were found using synthetic oligonucleotides rich in AT bases; in some cases, the opposite enantiomer of the product, with respect to that usually observed with st-DNA and copper alone, was detected. St-DNA shows a B-like conformation; hence, if a different conformation of DNA, depending on the sequence, is present, the copper bound-substrate might not reside in the same way as the B-DNA, leading to the attack from the other face of the enone moiety and to the formation of the opposite enantiomer of the hydration product. NMR studies with paramagnetic cations together with CD spectroscopy studies should be performed in order to identify the metal binding or localization site within the duplexes formed by the synthetic oligonucleotide and in order to explain why, in the presence of copper alone, it is possible to obtain both enantiomers of the product depending on the sequence of DNA.

4.4.3 CD- spectroscopy on DNA-sequences

The microenvironment provided by the DNA is a crucial factor in the catalysis. By using ligands of the second generation, a correlation between the conformation of DNA and the outcome of the Cu^{II}-catalyzed Diels-Alder reaction, in terms of enantioselectivity and rate acceleration, was observed.[33] It was found that sequences containing G-tracts which showed a B-type conformation but with a distortion towards A-DNA, were responsible for higher *ee* values and reactivity.[33] Therefore, to establish if a similar correlation existed also for the hydration reaction in the presence of the first generation ligands, the structure of some of the DNA-sequences screened in the catalytic experiments, was studied by using CD spectroscopy.

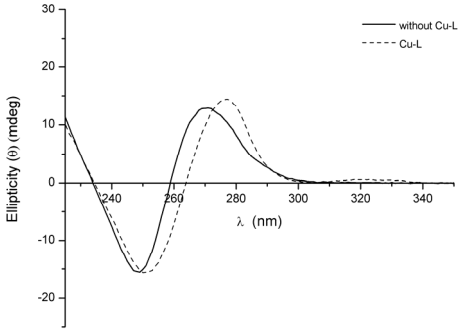
In Figure 6, the spectra of six representative oligonucleotides are shown. Although this screening does not involve a wide range of oligonucleotides, based on these spectra it seems that the sequences that gave moderate *ee* in catalysis show a profile (Figure 6, a, b, c) that is typical or close to B-DNA (that is a positive band centered near 275 nm, a negative band around 240 nm and a crossover centered near 258 nm).[38]

In contrast, the sequences which gave higher enantioselectivity (Figure 6 d, e, f) gave a CD spectrum that suggests the presence of a non-classical conformation of DNA. Interesting is the case of the sequences where five consecutive A/T bases are present (Figure 6, d and e); those gave the highest *ee* (82% and 77%, respectively) in the catalytic experiments. According to the CD spectra recorded for these sequences, the conformation of the resulting helix seems to resemble that of a A_2T triplex[38] with a positive band around 287 nm and a negative band around 245 nm. It has been shown that sequences rich in AT content, depending on the conditions of the environment, can adopt a non-classical DNA-conformation.[38]

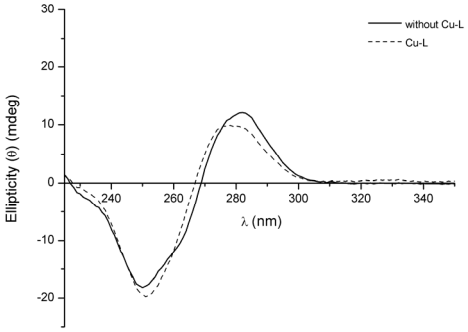
The presence of a triplex or another non-classical conformation of DNA, would imply a different mode of interaction of the ligand bound-copper with the DNA compared to the classical conformation of B-DNA.[39] This might be one of the reasons of the higher enantioselectivity observed in presence of those sequences, also considering that acridine molecules stabilize these types of structures.[40]

Figure 6. CD spectra of a) TATATATATATA (67% *ee* in D_2O); b) CGCGAATTCGCG (72% *ee* in D_2O); c) GCGCGCGCGCGC (50% *ee* in D_2O); d) CAAAAATTTTTG (82% *ee* in D_2O); e) TAAAAATTTTTA (77% *ee* in D_2O); f) TCAGTATACTGA (80% *ee* in D_2O) in MES buffer.

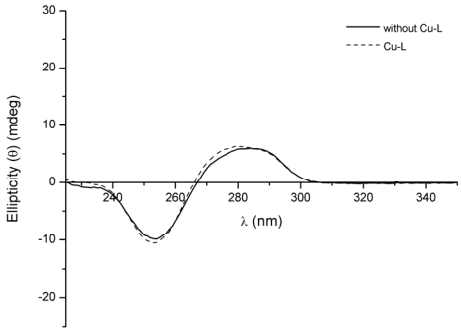
a)



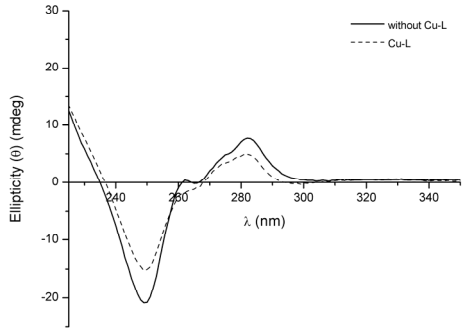
b)



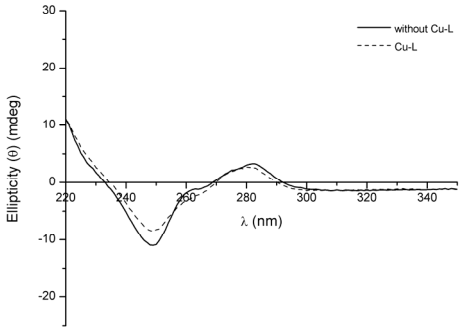
c)



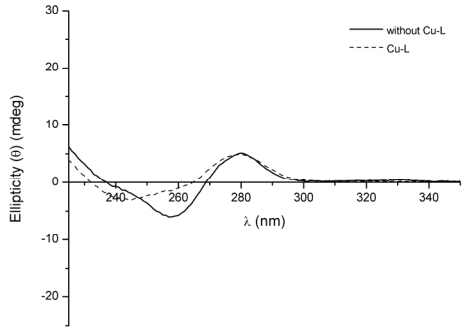
d)



e)

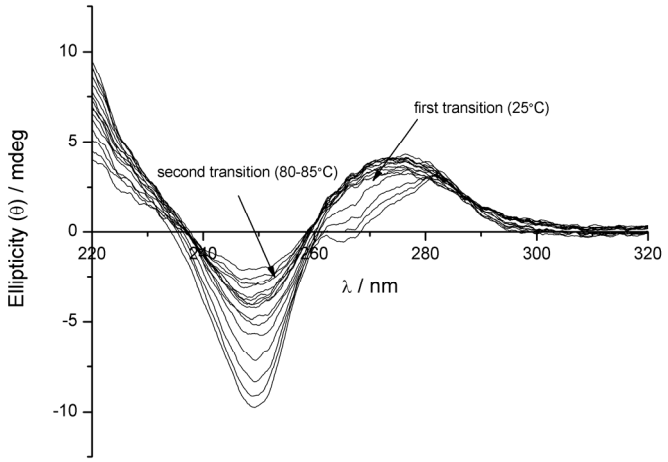


f)



We monitored the thermal denaturation behavior for the oligonucleotide CAAAAATTTTTG and indeed it seemed that two transitions are present (Figure 7).

Figure 7. Thermal denaturation followed by CD-spectroscopy for the oligonucleotide CAAAAATTTTTG.



From 5 °C to 22 °C the amplitude of the CD band at 282 nm increases and the band at lower wavelength (250 nm) becomes positive; from 25 °C to 80 °C the shape of the spectrum changes to that of a duplex structure and above 85 °C the CD spectrum appears to be consistent with a thermally disordered single-strand. Monitoring the melting at 284 nm,[38b] where it is possible to see only the transition associated with the triplex, a change in absorbance was detected, which would support this explanation. However, to date, such a high temperature for the second transition was observed for oligonucleotides longer than 12 bases or for 12-mer duplexes with a DNA hairpin.[38c] Hence, a broad range of sequences should be investigated in order to correlate the *ee* found in catalysis to the conformation of the DNA unequivocally.

4.5 Conclusions

In this chapter mechanistic insights on the *syn*-conjugate addition of water to α,β-acyl-imidazoles catalyzed by Cu^{2+}/L1-DNA is provided. Labeling experiments in deuterium oxide showed that the stereoselectivity of the reaction is substrate-dependent: with bulky substituents, performing the reaction in D_2O it was found that a single diastereoisomer is formed; an exception is represented by the substrate carrying a methyl group on the β-position (**4b**): in this case probably other processes are involved which led to the formation of both *syn*/*anti* products.

Based on the determination of the isotope effect and on the proton inventory which showed that only one proton is involved in the rate determining step of the reaction, a concerted mechanism was hypothesized. Efforts to improve the observed enantioselectivity showed that sequences rich in A/T consecutive bases or containing ATAT/TATA central segment are responsible for higher enantioselectivity in D_2O (up to 82%). Circular dichroism measurements on a small series of synthetic oligonucleotides suggests that high *ee* values are related to a non-classical conformation of the DNA. In presence of Cu^{2+} alone, both enantiomers of the product can be obtained depending on the DNA-sequence.

4.6 Experimental Section

General remarks:

Salmon testes DNA (st-DNA) was obtained from Sigma-Aldrich. Ligand L1 was described previously (see chapter 3). Substrates (**4a**, **b**, **d**) were prepared following known procedures.[41] The synthetic oligonucleotides were obtained from BioTez GmbH (Berlin). Deuterium oxide with a minimum isotopic purity of 99.9 atom-% of deuterium was purchased from Sigma-aldrich. ^{1}H-NMR spectra were recorded on a Varian 400 (400 and 100 MHz) in $CDCl_3$. Chemical shifts (δ) are denoted in ppm using residual solvent peak as internal standard (δ_H = 7.26 ppm for $CDCl_3$). Enantiomeric excess determination was performed by HPLC (LC20-AD from Shimadzu, Japan) analysis using UV-detection (Daicel-Chiralpak-AD 90:10 *n*-heptane/*i*-PrOH, 15 min, flow: 1 mL/min).

Physical methods, general remarks:

Circular Dichroism spectra were measured on a JASCO J-715 spectropolarimeter equipped with a temperature control attachment. The UV/Vis absorption spectra were measured on JASCO V-560 and JASCO V-570 spectrophotometers. The thermal denaturation behaviour of the oligo GAAAAATTTTTC was studied by CD spectroscopy. The spectra were recorded using a 5 °C temperature step.

Dissolution of synthetic oligonucleotides:

The lyophilized powder was dissolved in buffer (20 mM MES-H_2O, pH = 5.5 or 20 mM MES-D_2O, pD = 5.5). The solution was heated to 94 °C and slowly cooled to 5 °C, and left for 2 h at 0 °C prior to usage.

Reactions in the presence of synthetic oligonucleotides:

A 2 mL Eppendorf container was loaded with oligomer solution (400 µL, 2 mg/mL) in buffer (20 mM MES-H_2O, pH = 5.5 or 20 mM MES-D_2O, pD = 5.5), [Cu(L)$(NO_3)_2$] complex (200 µL, 0.3 mM final concentration), and 5 µL of a stock solution of substrate **4a** (4.6 mg in DMSO; 1 mM final concentration) at 5 °C. The reaction mixture was mixed by continuous inversion for 1 day at 5 °C. After extraction with diethyl ether, drying with Na_2SO_4, and concentration under reduced pressure, the products were analyzed by HPLC (Daicel-Chiralpak-AD 90:10 *n*-heptane/*i*-PrOH, flow: 1 mL/min).

Kinetics:

The aqueous solutions were prepared using deionized, doubly distilled water. Deuterium oxide was manipulated under a nitrogen atmosphere. The rate constants were determined by reversed phase HPLC (rp-HPLC) using caffeine as external standard and using 275 nm as wavelength for detection. The solutions were prepared immediately before use except in the study of the solvent isotope effect: in this case the solutions were left standing for 16 h at room temperature in order to allow the H/D balances to be reached.

Determination of isotope effect:

The experiments were conducted following the conversion of the substrate **4a** to **5a** in time in both H_2O and D_2O as solvents. The concentration of Cu/L1 used was 0.225 mM and 0.16 mM in substrate. The conversion was determined by np-HPLC (normal phase) (conversion = observed conversion × 1.109 – 0.4271). The reaction was stopped at different time points until 20% of conversion was reached; after extraction with diethyl ether, filtration over Na_2SO_4 and removal of the solvent, the samples were analyzed by np-HPLC (AD 90:10 *n*-heptane/*i*-PrOH, flow: 1 mL/min). The hydration reaction was considered a pseudo first-order reaction in **4a**, as the water concentration remains constant. A slight excess in Cu(II) compared with the

substrate was used, which allows to disregard the dissociation of the product from the catalyst as multiple turnovers are achieved. In the pre-equilibrium stage of the reaction (when the change in concentration of product formed is small), the backward reaction could be considered negligible. We can approximate [**4a**] with $[\mathbf{4a}]_0$ so the equation used to derive the k_{obs} was the following:

$$\frac{d[\mathbf{5a}]}{dt} = k_1 \cdot [\mathbf{4a}]_0 \qquad (1)$$

Plotting d[**5a**] versus t over the first 20% of the reaction give a curve with a slope corresponding to $k_1 \cdot [\mathbf{4a}]_0$. The observed apparent rate constant k_{obs} was obtained dividing the slope by $[\mathbf{4a}]_0$.

The kinetic isotope effect was calculated as the ratio between the rate constant for the reaction with the natural abundant isotope, and the rate constant for the reaction with the altered isotope and this is expressed by the equation (2):

$$\mathrm{KIE} = \frac{k_\mathrm{H}}{k_\mathrm{D}} \qquad (2)$$

Proton inventory:

Different mixtures of H_2O/D_2O were prepared, corresponding to a value of n (molar fraction D_2O) ranging from 0.08 - 1. The mixtures were left standing for 16 h at room temperature in order to allow the H/D balances to be reached. The concentrations used were 0.3 mM in copper and 0.22 in substrate; the reactions were stopped by quenching, with EDTA solution (1.2 eq. with respect to Cu^{2+}), after 26 h in order to get detectable conversion values for all the mixtures, and analyzed directly by rp-HPLC (reversed phase; Shimadzu LC10 HPLC, column AD-RH, 250 × 4,6 mm, particle size 5 µm, by Daicel; eluents: acetonitrile/water, gradient: 20/80 0 to 3 min, 30/70 at 4 min, 40/60 5 to 40 min , flow: 0.7 mL/min, T = 50 °C). The values of rates were determined using a correction factor of 1.1 for the external standard caffeine (obtained from a calibration curve). Caffeine was used as in a concentration of 0.2 mM. By plotting the ratio between the value of rates (ν_n, $M \cdot s^{-1}$) for each different mixture divided by the value of those ($\nu_{n,0}$, $M \cdot s^{-1}$) obtained for the reaction conducted in pure water versus the molar fraction of

deuterium in each mixture (*n*), a graph was obtained whose shape was indicative of the numbers of protons 'flying' during the rate determining step of the reaction.

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